



Characterization of viroids infecting grapevine in India

Pankhuri Singhal¹ · Shailender Kumar¹ · Richa Rai¹ · Sanjay Kumar Singh² · Virendra Kumar Baranwal¹

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Abstract

Several viroids infect grapevine worldwide. They are smallest infectious pathogens. Present study was undertaken to detect viroids in commercial grapevine genotypes in India. Four viroids viz., Grapevine yellow speckle viroid-1 (GYSVd-1), Grapevine yellow speckle viroid-2 (GYSVd-2), Australian grapevine viroid (AGVd) and Hop stunt viroid (HSVd) were detected in reverse transcriptase-polymerase chain reaction (RT-PCR) based assay. These viroids were found to present either alone or in different combinations. None of the samples showed the presence of citrus exocortis viroid. Few genotypes like Beauty Seedless and Black Mustard did not showed the presence of any of the five viroids. Thompson Seedless, Sharad Seedless and Black Prince were found to be associated with all the four viroids. Blast analysis of Indian GYSVd-1 isolate revealed a close identity of 99% with Q4-III isolate from Pakistan, and Indian GYSVd-2 isolate was found to have close identity of 100% with VB-108 isolate from Croatia. While Indian HSVd isolate showed close identity of 99% with SDLY-20 isolate from China and AGVd Indian isolate was found to have close identity of 99% with AGVd-Iran isolate. GYSVd-2 association with grapevine was found for the very first time from the Indian conditions. Nucleotide substitutions, additions and deletions were observed in the sequenced isolates of these viroids. Variation was observed in the secondary structure of Indian Hop stunt viroid (HSVd) isolate. Characterization of all the detected viroids indicated that they might have been introduced in India from Asian or European countries.

Keywords Viroids · Grapevine · RT-PCR · Phylogeny · Secondary structure

Introduction

Viroids are the non-coding subviral pathogens replicating autonomously in their hosts having small (241–401nts) circular single stranded RNA (Flores et al. 2005). They do not code for any proteins. Viroids are classified into two families, viz. *Pospiviroidae* and *Avsunviroidae* discriminating on the presence of a central conserved region in the secondary structure and nuclear replication (*Pospiviroidae*) or a branched secondary structure lacking the central conserved

region, presence of ribozymes and plastidial replication (*Avsunviroidae*). Though most viroids cause latent infection in the plants but some produce varied symptoms like stunting, epinasty, leaf distortion, localized veinal chlorosis and necrosis (Owens et al. 2012). Grapevine is mainly known to be infected by five viroids of the family *Pospiviroidae*, i.e., Hop stunt viroid (HSVd) (Sano et al. 1985) (genus *Hos-tuviroid*), Citrus exocortis viroid (CEVd) (Garcia Arenal et al. 1987) (genus *Pospiviroid*), Grapevine yellow speckle viroid 1 (GYSVd-1), Grapevine yellow speckle viroid 2 (GYSVd-2) (Koltunow and Rezaian 1988; Koltunow et al. 1989) and Australian grapevine viroid (AGVd) (Rezaian 1990) (all belonging to the genus *Ap-scaviroid* and occurring exclusively in grapevine). Recently two other viroids, Grapevine latent viroid (GLVd) of genus *Ap-scaviroid* and Grapevine hammerhead viroid-like RNA (GHVd) (unclassified) were also detected to infect grapevine (Gambino et al. 2014). While GYSVd-1 and HSVd have worldwide distribution, GYSVd-2, AGVd and CEVd are found sporadically. GYSVd-1 and GYSVd-2 are reported to cause grapevine yellow speckle disease characterized by symptoms such as

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✉ Virendra Kumar Baranwal
vbaranwal2001@yahoo.com

¹ Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India

² Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India

yellow speckle under high temperature greenhouse conditions from Australia. These viroids may also cause vein banding in grapevine plants co-infected with Grapevine fan leaf virus (GFLV) (Krake and Woodham 1983; Szychowski et al. 1995). HSVd, CEVd and AGVd are known to cause latent infection in grapevine plants not producing any symptoms. However, latently infected plants may serve as inoculum sources for other susceptible hosts, as suggested for HSVd and CEVd infected grapevine that were the likely source of epidemics in hop and citrus (Kawaguchi-Ito et al. 2009).

In earlier studies from India, detection of grapevine viroids has been attempted and GYSVd-1 and HSVd were reported from the Karnataka (Sahana et al. 2013). Viroid AGVd has also been reported from the southern states like Karnataka, Tamil Nadu and Maharashtra (Adkar-Purushothama et al. 2014). There have been no reports of GYSVd-2 and CEVd infection from the commercial grapevine cultivars in India. Moreover, a rigorous study of the infection by these viroids from the commercial grapevine cultivars from India and their diversity analysis has not been attempted till date. Therefore, a study was undertaken to assess the occurrence and characterization of five viroids in grapevine cultivars including commercial ones collected from different places in India was to be evaluated in order to find out their diversity as well as phylogenetic relationship.

Materials and methods

Sample collection and RNA extraction

Grapevine leaves showing typical symptoms of viroid infection in 19 grapevine genotypes grown in Germplasm Block of Indian Agricultural Research Institute (IARI), New Delhi were collected during May–November, 2017. Three more commercial grapevine cultivars originally collected from Maharashtra and Imphal maintained in glasshouse facility at Division of Plant Pathology, IARI, New Delhi, showing viroid or virus suspected symptoms such as red leaf or leaf roll symptoms were also included in the present study for confirmation of the presence of viroid in them. Healthy grapevine plant maintained in the glasshouse was used as negative control. Yellow speckle symptoms characteristic of Grapevine yellow speckle disease were observed in only genotype Sharad Seedless. About 200 mg of leaf sample was used for the isolation of total RNA using Qiagen RNeasy plant mini kit based on the method specified by Mackenzie et al. (1997) and its quality and quantity were assessed using NanoDrop™ One spectrophotometer (ThermoScientific, Wilmington, USA). The RNA was stored at -80°C until further use.

Reverse transcriptase-polymerase chain reaction

The presence of any of the five viroid infection namely GYSVd-1, GYSVd-2, AGVd, HSVd and CEVd, in all the grapevine cultivars collected was confirmed through RT-PCR assay carried in two steps using each viroid specific degenerate primer pairs. Each viroid specific degenerate primer pair was designed on the basis of full genome sequence available of viroids in NCBI database (Table 1). Single-stranded cDNA was synthesized from approximately 500 ng of the purified RNA of each sample using each viroid specific reverse primer and the Improm-II Reverse Transcriptase (Promega, Madison, USA), followed by PCR amplification of full genome from the cDNA using each viroid specific primer pair. The PCR mixture contained 0.25 μM dNTP mix, 1X reaction buffer, 4 μl of cDNA template and 1 unit of DyNAzyme II DNA polymerase (Thermo Fisher Scientific, Madison, USA), in a total volume of 25 μl . The PCR conditions for each viroid-specific primer pair was used as given in Table 2.

Cloning and sequence analysis

The specific amplicons were purified using the Wizard[®] SV Gel and PCR Clean-up System (Promega, USA) and cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into DH5 α strain of *Escherichia coli* (Stratagene, USA). The transformed colonies were confirmed through colony PCR and characterized through restriction digestion using *EcoRI*, *Not I*. At least two clones of each amplicon were sequenced in both directions using SP6 and T7 universal primer pair. Sequences were assembled to generate the consensus sequence of the respective genes using *BioEdit* Sequence alignment editor (version 7.2.6.0). Edited nucleotide sequences were searched for homologous sequences through nBLAST analysis on NCBI (<http://www.ncbi.nlm.nih.gov/>). Consensus sequences were aligned using CLUSTALW software and used for the construction of phylogenetic tree through neighbor-joining method using MEGA 7.0 version software (Kumar et al. 2016). The robustness of tree topology was evaluated with 1000 bootstrap resamplings. The possible secondary structures were predicted at a folding temperature of 37°C using RNA structure 6.0 web server and the conserved domains were located (Giguere and Perreault 2017).

Table 1 Sets of primers used to amplify the selected grapevine viroids

Tested viroid	Primer name	Sequence (5′–3′)	Positions	Expected cDNA (bp)	References
GYSVd-1	341M	CACTCGCGGGGCGCGTTGGA		366	Sahana et al. (2013)
	342P	CAATCCCCGGAACCCCGCT			
GYSVd-2	GYSVd-2-P1	ACTAGTACTTTCTTCTATCTCCGAAG	188–208	375	Jiang et al. (2009)
	GYSVd-2-P2	ACTAGTCCGAGGACCTTTTCTAGCGCTC	166–187		
HSVd	HSV-78P	AACCCGGGGCAACTCTTCTC	76–95	303	Sahana et al. (2013)
	HSV-83M	AACCCGGGGCTCCTTTCTCA		234	
AGV	AGV-60RT	TTTTTCTTCCTAGCTTCGCG		330	Adkar Purushotam et al. (2014)
	AGV-77F	TAAGACTCACCTGGCGACTC			
	AGV-37R	TCGGTGAGTACCACAGGAAC			
CEV	CEVd-mF	GTGTCCTTCCTTCGCTGCTG	182–202	153	Hajizadeh et al. (2012)
	CEVd-mR	TGGCCCGGAGAACAGTGAAG	315–334		
	F1	CGGGATCTTTCTTGAGGTCC	1–21		Designed primers in this study
	R1	GAACCCTAGATTGGGACCCT	350–369	369	
	R2	CCGCTTTTCTTATATCTTCACTG	300–322	322	

Results

Identification and characterization of viroids

Among the five viroids analyzed only four were found positive either individually or in mixed infection in the tested samples. CEVd was not detected in any of the tested samples. In this study, out of twenty-three cultivars tested for the presence of the grapevine viroids, fifteen were found to be infected by one or more than one viroid. In genotypes like Anab-e-shahi (HSVd), chardonnay (GYSVd-1) and JM, Syrah (AGVd) presence of only one viroid was observed. In Thompson seedless, Sharad seedless and Black prince genotypes presence of all the four viroids was observed. None of the five viroids were tested positive in seven varieties viz. Tempronillo, Syrah R4,P1, Syrah R3,P5, Black Mustard, Bharat early*Syrah, Bharat early and beauty seedless (Table 3).

Grapevine yellow speckle viroid-1

Sequencing of GYSVd-1 was done from two Indian cultivars (Thompson Seedless and Chardonnay). Sequence of 367 bp (Gen Bank Accession No. MH476216) was obtained from Thompson Seedless (Maintained in Glass house) and blast analysis of the sequence revealed 99% sequence identity with the sequence of GYSVd-1 complete genome (KY978404.1). Nucleotide sequence variations were observed at G⁹² → A, T³³⁴ → A and A³³⁷ → G. The amplicon from another variety (Chardonnay, New Delhi) was of 366 bp (GenBank Accession No. MH476217). Blast analysis of the sequence revealed identity of 100% with the Grapevine yellow speckle viroid 1 isolate DgSV1-8 (MF576407)

from Nigeria at nucleotide level. Also, a sequence identity of 95% was observed between both the Indian isolates from Thompson Seedless and Chardonnay.

Though the sequence identity of only 85% was obtained with the reference sequence of type strain (NC_001920) but on sequence comparison it was revealed that the sequence variations occurred mainly in variability (V) domain and terminal left (TL) domain. The Central conserved region (CCR) was similar to that of the other members of the genus *Apscaviroid* (positions 92–107 and 254–268). The portions of the terminal right (TR) region which is more or less conserved in the members of *Apscaviroid* having genome size greater than 300 nts, showed more differences at the sequence level than at the structural level (Fig. 1a).

Phylogenetic relationship of Indian GYSVd-1 isolates with globally 13 variants was analyzed by neighbor-joining method. The variants clustered in four separate clads. GYSVd-1 Indian isolate clustered in a clad with variants from the Asian countries like Pakistan (KY978404), Iran (KF916042), Thailand (AY639606) and China (KX966269). GYSVd-1 Indian isolate was found to be 94% identical with GYSVd-1 Kar-1 (AB742222) reported from Karnataka which clustered in a different clad with isolates from Japan (AB028466) and South Korea (KY244035). The isolates from Canada (AF462157), Italy (EU682454), Croatia (MF979527) and Czech Republic (KT000346) clustered in a single clad while the variant from USA (KF137564) clustered in a completely different clad singly (Fig. 2a).

The presence of GYSVd-1 was observed in 7 out of 22 tested genotypes, i.e., 30% of the plants were found infected (Table 3).

Table 2 PCR profiles of the five tested grapevine viroids

Viroid primer pair	Initial denaturation		Denaturation		Annealing		Extension		Elongation	
	Temp. (°C)	Time (min)	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)	Temp. (°C)	Time (min)
GYSVd-1/342P F/341 M R	94	4	94	30	63	30	72	30	72	10
GYSV-2 P1 F/P2 R	94	4	94	30	56	30	72	30	72	10
HSVd 83R/78PF	94	4	94	30	57.4	30	72	30	72	10
AGVd 77F/37R	94	4	94	30	57	30	72	30	72	10
CEVd mF/mR	94	4	94	30	51	30	72	30	72	10
CEVd F1/R1 F1/R2	94	4	94	30	46	30	72	30	72	10
No. of cycles	1		30							1

Table 3 Presence of different viroid(s) in grapevine genotypes

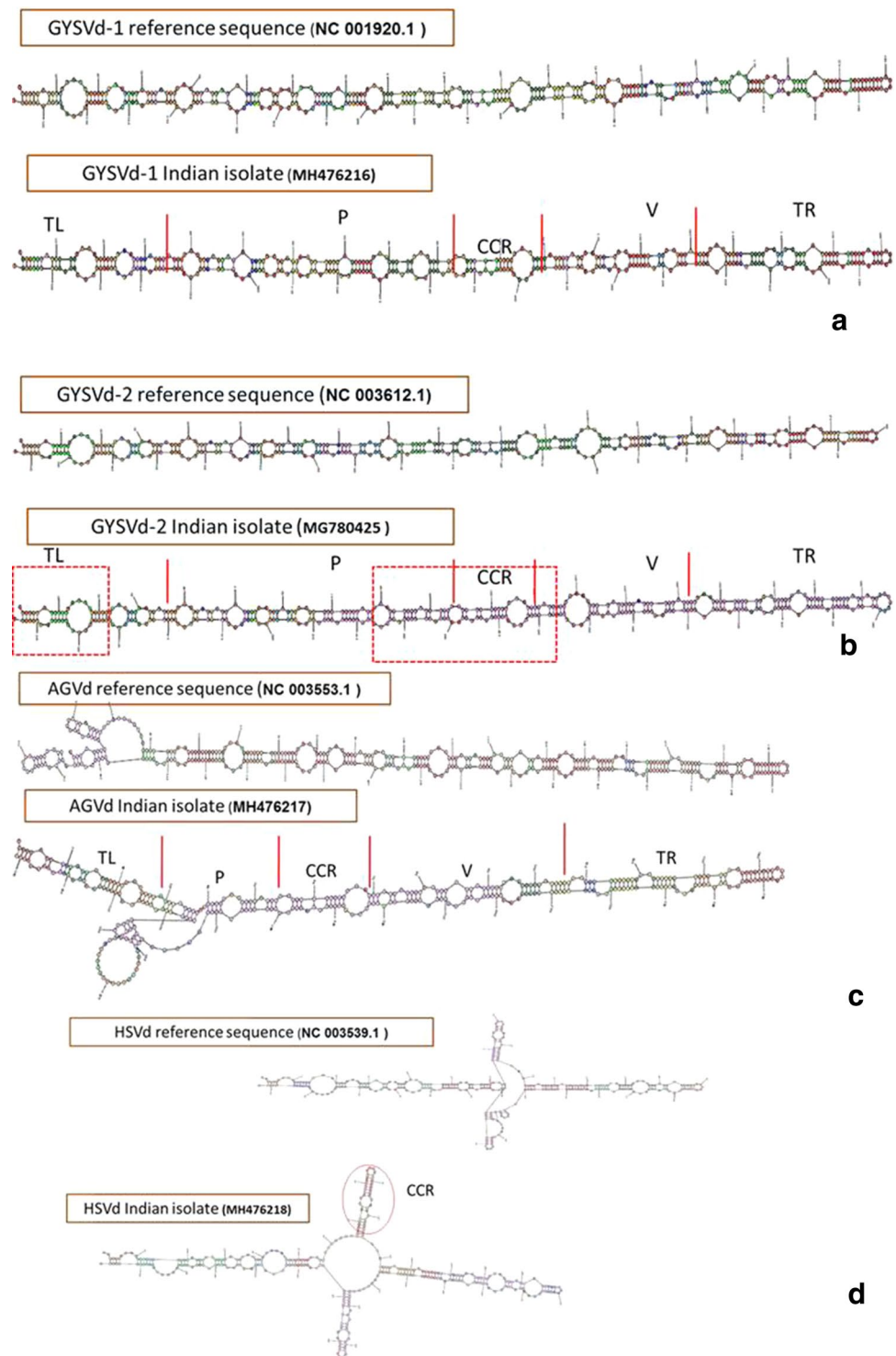
S. no.	Genotype	GYSVd-1	HSVd	AGV	GYSVd-2
Glasshouse maintained genotypes from Nashik, Maharashtra and Imphal and Manipur					
1	Thomson seedless	Positive	Positive	Positive	Positive
2	Flame seedless	Positive	Positive	–	Positive
3	Sharad seedless	Positive	Positive	Positive	Positive
Vineyard- Grape Germplasm Block of Indian Agricultural Research Institute					
4	Chardonnay (R4, P5)	Positive	–	–	–
5	Tempranillo (R5, P6)	–	–	–	–
6	Syrah (R4, P1)	–	–	–	–
7	Syrah (R3, P5)	–	–	–	–
8	Syrah (R4, P16)	–	–	Positive	–
9	NRC grape genotype	Positive	Positive	Positive	–
10	Julesky Muscat	–	Positive	–	–
11	Black Muscat	–	–	–	–
12	Hur	–	–	Positive	Positive
13	Perlette	–	–	Positive	–
14	Pusa Urvashi	–	Positive	Positive	–
15	Pusa Navrang	–	Positive	–	Positive
16	Syrah (R2, P5)	–	positive	–	–
17	Syrah (R2, P6)	Positive	–	Positive	–
18	Bharat early × Syrah	–	–	–	–
19	Anab-e-shahi	–	Positive	–	–
20	Black prince	Positive	Positive	Positive	Positive
21	Bharat early	–	–	–	–
22	Beauty seedless	–	–	–	–

Grapevine yellow speckle viroid-2

GYSVd-2 was sequenced from isolate SS-N of cultivar Sharad Seedless (Nashik, Maharashtra) and whole genome size of 375 bp (MG780425) was obtained. Blast analysis of the sequence revealed close identity of 100% with Grapevine yellow speckle viroid 2 isolate VB-108 Cof Croatia (MF979530). The Indian isolate was 12 nucleotides larger in size at positions 187–198 nts.

The sequence of GYSVd-2 Indian isolate was compared with the sequence of the type strain (NC_003612) to identify new polymorphisms. It was found to be 12 nucleotides more than the reference sequence in size. A sequence identity of 97% was observed between the strains. A gap of 12 nucleotides was observed in case of type strain (positions 187–198 of Indian isolate) and mutation was found at position A³⁰⁰ → G³¹². Though the sequences were more or less completely conserved in most domains including TL, Pathogenicity (P) and CCR, structural variations in

Fig. 1 Predicted secondary structures of the Indian isolates of viroids sequences in the present study and their reference sequences through RNA structure 6.0. Lines differentiating the five domains in the secondary structure. Terminal left (TL), P (pathogenicity), central conserved region (CCR), V (variability) and terminal right (TR). **a** Secondary structure of grapevine yellow speckle viroid-1 (GYSVd-1) reference sequence and Indian isolate. **b** Secondary structure of grapevine yellow speckle viroid-2 (GYSVd-2) reference sequence and Indian isolate. **c** Secondary structure of Australian grapevine viroid (AGVd) reference sequence and Indian isolate. **d** Secondary structure of hop stunt viroid (HSVd) reference sequence and Indian isolate



the secondary structure could be observed between the two strains in V and TR domains. The secondary structure of VB isolate was similar to type strain (Fig. 1b).

Phylogenetic relationship of GYSVd-2 variants was analyzed by neighbor-joining method using eight variants

of different countries. GYSVd-2 Indian isolate was found to be clustered in a clad with other variants from the Asian countries like Pakistan isolate (KY978405), South Korea (LC177112), Thailand (KP010021) as well as an isolate from Croatia (MF979530). Other isolates

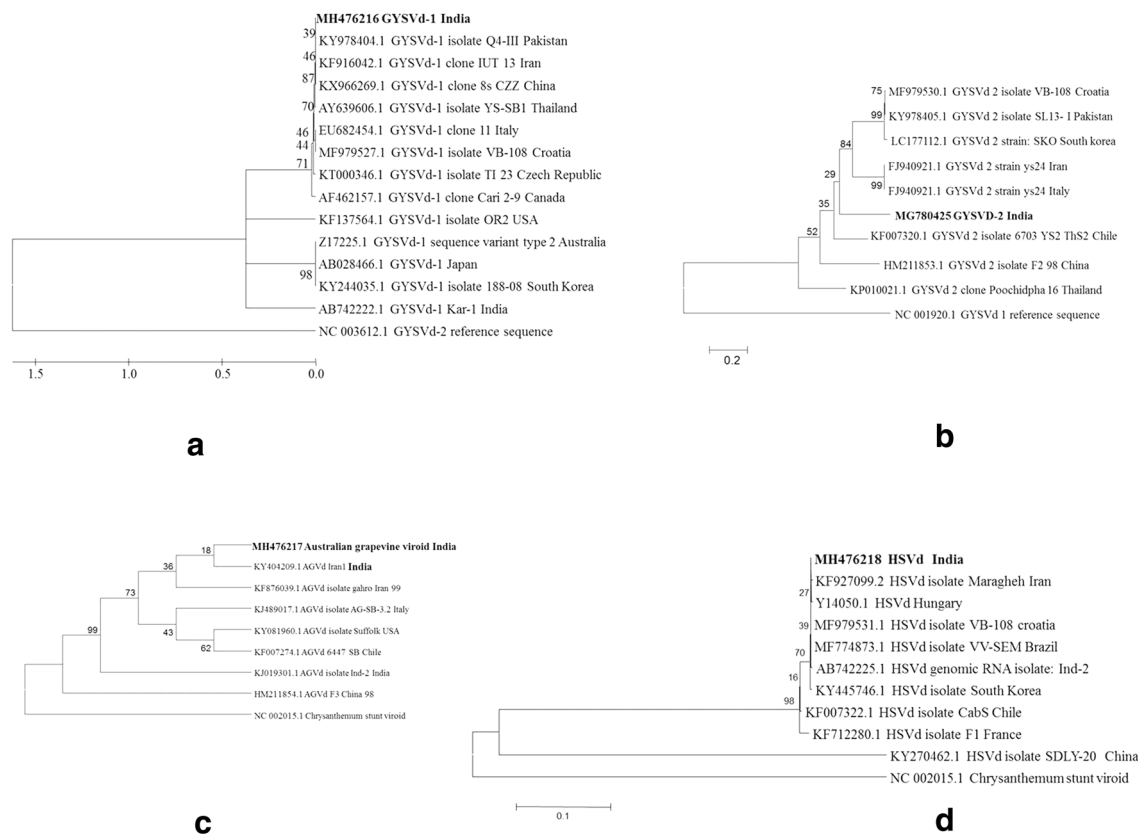


Fig. 2 Phylogenetic tree constructed by MEGA 7 version using neighbor joining method bootstrapped at 1000 replications. **a** Construction tree based on the alignment of nucleotide sequences of 14 GYSVd-1 isolates of different countries. Grapevine yellow speckle viroid-2 (GYSVd-2) reference sequence was used as outgroup. **b** Construction tree based on the alignment of nucleotide sequences of 10 GYSVd-2 isolates of different countries. Grapevine yellow speckle

viroid-1 (GYSVd-1) reference sequence was used as outgroup. **c** Construction based on the alignment of nucleotide sequences of eight AGVd isolates of different countries. **d** Construction based on the alignment of nucleotide sequences of ten HSVd isolates of different countries. **c, d** Chrysanthemum stunt viroid reference sequence was used as outgroup. Bar = number of substitutions per site

from Iran (FJ940921), China (HM211853) and Italy (FJ940921) clustered in a separate clad in the phylogenetic tree (Fig. 2b).

The presence of GYSVd-2 was observed in 8 out of 22 tested genotypes (Table 3).

Australian grapevine viroid

AGVd was cloned from the amplicon of desired size of 331 bp (MH476217) from Thompson Seedless observed on the gel which was further eluted, cloned and sequenced. Blast analysis of the sequence revealed close identity of 99% with Iran isolate (KY404209) at nucleotide level. Sequence variation was observed at position 241 where T nucleotide was missing in the Iran isolate. The sequence of the Indian isolate was also compared with the reference sequence of the type strain (NC_003553) which is a complete genome of 369 nucleotides. A sequence identity of 99% was found between the two with mutations at $C^{246} \rightarrow \text{nil}$, $A^{276} \rightarrow G$,

$C^{278} \rightarrow T$, $T^{283} \rightarrow \text{nil}$, $T^{310} \rightarrow A$. In spite of the high sequence identity, a difference was seen in the secondary structure of the Indian isolate and the type strain in the TL and the P domains. While the structure of the CCR (positions 95–110 and 263–279), a characteristic of all the members of genus *Apscaviroid* was found to be same. The secondary structure of the Iran isolate was found to be rod shaped with no side loops and differences were observed in the regions of TL and P domains in the structure from both Indian isolate and reference sequence (Fig. 1c).

Phylogenetic relationship of AGVd variants was analyzed by neighbor-joining method using seven variants of different countries. AGVd Indian isolate was found to be clustered in a clad with other variants from the Asian countries Iran isolate (KY404209). Also the Indian isolate was found to be different from the AGVd isolate Ind-2 (KJ019301) reported from the Maharashtra and Karnataka region earlier which clustered in a separate clad with the China isolate

F3 (HM211854). Another clad was observed in the phylogenetic tree comprising of isolates from USA and Chile (Fig. 2c).

The presence of AGVd was observed in 9 out of 22 grapevine genotypes tested (Table 3).

Hop stunt viroid

A desired amplification was obtained using 83R/78PF primer set which was further cloned and sequenced and known to be the size of 306 bp (GenBank Accession No. MH476218) from F-Pachore vani of cultivar Flame Seedless. Blast analysis of the sequence revealed close identity of 99% with Hop stunt viroid isolate SDLY-23 (KY270463) from China at nucleotide level. Sequence variation was observed at position G⁹⁷ → A and 293 where a nucleotide was found missing in the China isolate. In spite of the high sequence identity there was a marked difference in the secondary structure of both the isolates. Though the CCR region, a characteristic of *Hostuviroid* could be located in the secondary structure. The sequence identity of HSVd Indian isolate with reference sequence of the type strain (NC_001351) was found to be 96% with sequence variations at positions A¹ → G⁷⁶, A² → C⁷⁷, G⁷⁶ → T¹⁵³, C⁷⁷ → G¹⁵⁴, T¹¹⁸ → C¹⁹⁶, G¹⁹⁰ → A²⁷⁰. The secondary structure of the reference sequence was found to be different from the Indian isolate except in the CCR and Terminal Conserved right (TCR) domains (Fig. 1d).

Phylogenetic relationship of HSVd variants was analyzed by neighbor-joining method using nine variants of different countries. HSVd Indian isolate was found to be clustered in a single clad with all the other nine variants of HSVd taken in analysis like the Croatia isolate (MF979531) and Brazil isolate (MF774873) of HSVd. It was found to be clustered in a single clad with that of the HSVd Ind-2 (AB742225), other isolate reported from India from the Karnataka region (Fig. 2d).

The presence of HSVd was found in 10 out of 22 samples tested recording highest among all the other viroids (Table 3).

Citrus exocortis viroid

The presence of CEVd was tested for in all the glasshouse samples first with published primer set, i.e., CEVd mF/CEVdmR at annealing temperature 51 °C. No amplification was obtained in any of the tested grapevine genotypes. To confirm the results two more degenerate primer sets were designed comparing the CEVd-g isolate and the Indian CEVd citrus isolate, i.e., CEVd F1/R1 and CEVd F1/R2 and tested at gradient annealing temperature between 46–48 °C using Flame seedless and Sharad seedless and negative control (water). Amplification of ~ 300 bp was obtained in case of both the cultivars. The gel was eluted and sequenced.

Blast analysis revealed that the obtained sequence did not match with any of the CEVd isolate and it was a non-specific amplification. Thus, the samples were deemed to be devoid of CEVd (data not provided).

Discussion

Grapevine is the fifth most important fruit crop in India in terms of production but a number of viruses and viroids are known to cause reduction in quantity and quality of the produce. Most of these viroids do not produce any symptoms except GYSVd-1 and GYSVd-2, which produce yellow speckle and vein banding symptoms but only at higher temperatures or in late summers in Australia (Taylor and Woodham 1972). In our study we did not find any symptoms of both GYSVd-1 and GYSVd-2, probably because these samples had infection by grapevine leaf roll viruses (GLRaVs) which might have masked the symptoms of the two viroids. Further, these varieties are found in mixed infections of viruses and viroids due to which distinguishing the symptom of viroids was difficult. Though reddening symptoms were observed in some varieties like Black Prince under field but they cannot be attributed to viroid infection.

GYSVd-1 Indian isolate was found to have one nucleotide more than the reference sequence. GYSVd-1 has been reported previously from Karnataka, the southern part of India (Sahana et al. 2013) and it did not cluster together with Indian isolate from Imphal, Manipur though both have been cloned from same genotype. This difference in identity between the two isolates could either be attributed to introduction of same genotype (Thompson Seedless) from different places or mutations and recombination in the same genotype with time and place. The genome size of GYSVd-2 Indian isolate was 12 nucleotides more than that of the reference sequence. It was reported for the first time from the Indian conditions from Nashik, Maharashtra (Singhal et al. 2019).

The presence of GYSVd-1 was found least (30%) among all the four though it has a high worldwide occurrence infecting the grapevines of Japan, Germany, Australia, Turkey, Tunisia, USA, New Zealand with recent reports from Iran (Hajizadeh et al. 2012), China and Japan (Jiang et al. 2012). While, the presence of GYSVd-2 was found comparatively higher recorded from 35% of the tested samples in Indian vineyards. Though its worldwide occurrence is less reported from few places like Italy, Turkey, China, and recently reported from Iran (Hajizadeh et al. 2012), Italy (Gambino et al. 2014) and Nigeria (Zongoma et al. 2018) with low incidence. However, GYSVd-1 and GYSVd-2 have a restricted host range only host being grapevine.

The genome size of HSVd Indian isolate was four nucleotides more than that of the reference sequence. The presence of HSVd was found maximum among all viroids as 43% of the tested genotypes were found positive. HSVd is reported to have a worldwide occurrence recorded from the grapevines of Japan, Germany, Australia, USA, Turkey, with recent reports from Iran (Hajizadeh et al. 2012), China and Japan (Jiang et al. 2012), Central Washington state (Kappagantu et al. 2017). It has also been reported from a wide range of hosts including hop (Sasaki and Shikata, 1977), peach, apricot, plum (Sano et al. 1989), cucumber (Sano et al. 1984), citrus (Sano et al. 1988), almond (Kofalvi et al. 1997), jujube (Zhang et al. 2009) and mulberry (Elbeaino et al. 2012) apart from grapevine.

The genome size of AGVd Indian isolate was 38 nucleotides less than that of the reference sequence. AGVd had previously been reported from Karnataka and Maharashtra region (Adkar-Purushothama et al. 2014) but it did not cluster together with Indian isolate from Maharashtra and Delhi. AGVd was found positive in 39% of the tested genotypes. The previous report from India suggest low infection rate of AGVd from India (9.3% infection rate). Previously, AGVd has been reported from the vineyards of Australia, China, USA, Tunisia, Mediterranean region, including recent reports from Iran (Hajizadeh et al. 2012), Italy (Gambino et al. 2014) and Turkey (Buzkan et al. 2018).

The phylogenetic analysis of all the four viroids indicated that they might have been introduced in India from other Asian, European or other South American countries through the introduction of infected propagative material from these countries, since these viroids lack vector transmission.

Since, the viroids do not code for any proteins, their mode of action is mainly through the functional domains found in the secondary structure of the viroids (Riesner et al. 1983). Therefore, we prepared the secondary structure of the detected viroids and located all the functional domains and observed that the central conserved domain was similar for all the viroids of the genus *Apscaviroid* (GYSVd-1, GYSVd-2 and AGVd). The CCR of these three viroids was distinct from HSVd belonging to genus *Hostuviroid* proving and this has been used for placing these viroids in distinct genera of the family *pospiviroidae* and their classification. Though the terminal conserved regions (TCR) were found to be more or less similar but since the same is not found in the lower strand thus it is not used to classify species (Giguere and Perreault 2017). The sequence of the Indian isolates were compared with the sequence of type strain to identify mutations and polymorphisms. The effects of identified mutations, especially novel mutations affecting the conformation of the secondary structure were examined. Though the loops associated with important biological functions of the viroids were found to be conserved in the variants. (Qiu et al. 2016). Still the secondary structure of HSVd Indian

isolate was not linear as the type strain and was found to be folded in a different conformation.

The genotypes which were found to be singly infected by one viroid could be further used to study the symptom of viroid infection. Though GYSVd-1, HSVd, and AGVd were found to infect grapevine singly but presence of GYSVd-2 was always found in different combinations with other viroids. In earlier studies Anab-e-shahi was reported to be infected by both HSVd (Sahana et al. 2013) and AGVd (Adkar-Purushothama et al. 2014) whereas in our study presence of only HSVd could be detected. This may be due to the introduction of the same genotype from different places in the form of propagation material. This only indicates that screening of the propagated material before introduction should be of prime importance. It was also noted that the leaves of same genotype (Syrah) collected from different plants from the same block showed different combinations of viroids. Few Syrah plants were found to be completely free of presence of any viroid, while few plants either showed the presence of only AGVd or HSVd, while few were even found to be infected by both GYSVd-1 and AGVd. This could be probably due to the introduction of several roostocks of same genotypes as different accessions. The genotypes which were found to be free from infection by all the five viroids can be used for the production of quality planting material and can also be used to study the host-viroid interactions. It only indicates that we have to screen our commercial genotypes for freedom from viroids.

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